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71 Applicant: ABBOTT LABORATORIES

Abbott Park Illinois 60064(US)

72 Inventor: Wong, Martin  
30947 Blackhawk Trail  
Grayslake Illinois 60030(US)

74 Representative: Modiano, Guido et al  
MODIANO, JOSIF, PISANTY & STAUB  
Modiano & Associati Baaderstrasse 3  
D-8000 München 5(DE)

54 Preparation of sized populations of liposomes.

57 The present invention provides novel methods for generating aqueous liquid encapsulating multilamellar lipid vesicles (liposomes) wherein one or more lipids, or lipid conjugates, and an aqueous liquid to be encapsulated, are agitated in a vessel in the presence of spherical contact masses having a mean diameter of less than 3,000 microns, the preferred size range being 50-100 microns in diameter, and resulting in a substantially homogeneous population of vesicles having diameters in the range of about 150 to about 3,000 nanometers. The methods allow for the use of small quantities of marker and lipid, leave no residual solvents, allow for contact only with glass surfaces, and involve no transfer of liposome preparations from lipid film drying vessels to sizing apparatus.

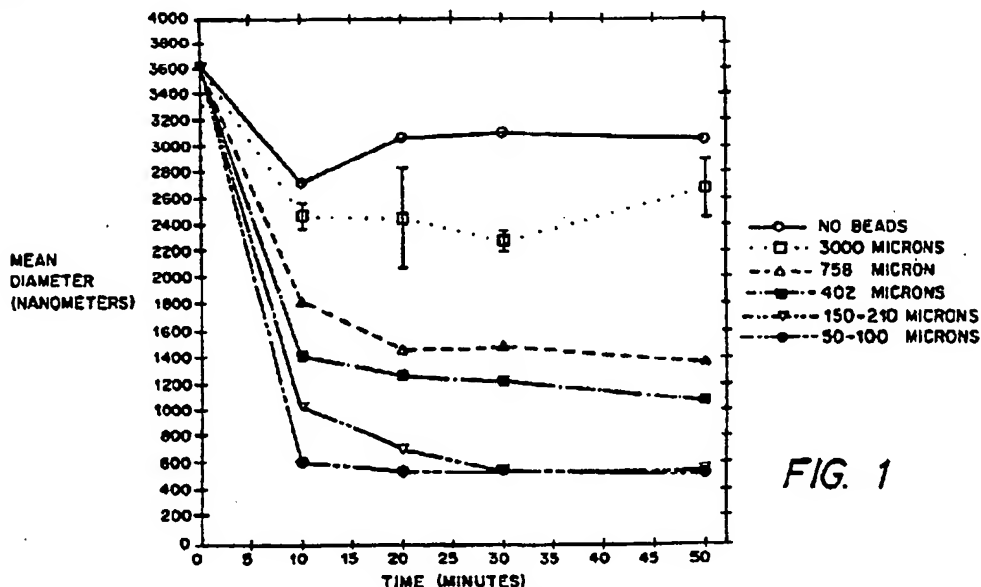


FIG. 1

## PREPARATION OF SIZED POPULATIONS OF LIPOSOMES

BACKGROUND OF THE INVENTION

5 The present invention relates generally to a method for the preparation of uniformly sized populations of lipid vesicles, liposomes, which may be used to encapsulate aqueous materials.

Liposomes are spherical shells of amphipathic molecules which isolate an interior aqueous space from the bulk exterior aqueous environment and are characterized by their lipid composition, the most commonly used lipid component being phospholipid. Because liposomes can be made to contain hydrophobic  
10 molecules within their membrane, or hydrophilic markers within their internal aqueous space, or both, liposomes can be used as potential vehicles for the delivery of drugs in vivo and as the basis for immunoassay systems in vitro such as the Liposome Immuno-Lytic Assay (LILA) which involves the antibody-triggered complement-mediated lysis of liposomes. For use in such complement-mediated immunoassays, liposomes should be homogeneous in size distribution, be small enough to remain in  
15 suspension, and be large enough to entrap sufficient marker, such as fluorophore or enzyme, to provide a high signal.

Many methods have been described in the literature for making a wide variety of both multilamellar and unilamellar liposomes. See, Szoka, et al., Annu. Rev. Biophys. Bioeng., 9:487-508 (1980); Deamer and Uster, "Liposomes" Ch. 1, Liposome Preparation: Methods and Mechanisms; Marc J. Ostro (Ed.) M. Dekker, Inc., pp. 27-51 New York, NY (1983); and Szoka, et al., Proc. Nat'l. Acad. Sci. U.S.A., 75:4194-4198 (1978).

Multilamellar vesicles (MLV) are prepared by depositing lipids from organic solvents in a thin film on the wall of a round-bottom flask by rotary evaporation under reduced pressure followed by hydration with aqueous buffers and agitation. Bangham, A., et al., J. Mol. Biol., 13:238-252 (1965). The hydration time,  
25 method of resuspension of lipids, and thickness of the lipid film can result in markedly different preparations of MLVs, despite a constant lipid concentration and composition, and volume of suspending aqueous phase. The size ranges obtainable by this method vary from 0.4 to 50 microns (400 to 50,000 nanometers). Papahadjopoulos and Miller, Biochem. et Biophys. Acta., 135:624 (1967). Szoka, F., et al., Proc. Nat'l. Acad. Sci. U.S.A., 75:4194-4198 (1978).

30 Mezei, et al., U.S. Patent No. 4,485,054, disclose a procedure for producing large multilamellar lipid vesicles (MLV) which may be used to encapsulate biologically active materials, particularly lipophilic substances. The lipid film forming step is conducted in a vessel partially filled with inert, solid, contact masses, in particular, spherical contact masses having a diameter of 1.0 mm to 100 mm (1,000 to 100,000 microns) and results in liposomes reported to range in diameter from 5 to 10 microns (5,000 to 10,000  
35 nanometers).

MLVs are relatively easy to prepare, can be made from a wide variety of lipid compositions, and are efficiently lysed by complement. Because they have many lamellae, they are stable against leakage and therefore have good shelf life. However, the use of MLVs can be disadvantageous because MLV preparations typically are heterogeneous in size distribution, in shape, and in entrapped volume. For in vivo  
40 applications such as drug delivery, the largest MLVs are most readily filtered from the blood stream and consequently most rapidly sequestered in the lungs and reticulo-endothelial system, thereby limiting the circulating half-life of liposome-entrapped drugs and hindering efforts towards tissue specific drug targeting. Scherphof, et al., Biochem. Soc. Trans., 15:345-348 (1987); and Juliano and Stamp, Bioch. Biophys. Res. Comm., 63:651-658 (1975).

45 When used in complement-mediated assays, the largest MLVs tend to settle, thereby changing the distribution of MLVs in solution and causing a shift in the immunoassay standard curve. Further, liposome lysis varies as a function of both the size and the type of liposome used in the immunoassay. Richards, et al., Biochem. et Biophys. Acta., 855:225-230 (1986). Liposome interaction with plasma also varies as a function of the size and type of liposome. Scherphof, et al., Liposome Technology, Volume III, pp. 205-224 Edited by G. Gregnolia, CRC Press Boca Raton, Florida (1984). These results indicate that liposome size is  
50 an important parameter which needs to be controlled in order to obtain reproducible complement mediated lysis.

Although the heterogeneous size problem can be overcome by sizing by passage through filters, the filters tend to clog when high concentrations of lipid and large volumes of liposomes are filtered. Clogging is also a function of the lipid composition, e.g., the charge of the lipids used to prepare the liposomes, the

interaction with the filter material, and the tendency of the liposomes to aggregate during and after filtration. Frequent changes of filters is both difficult and inconvenient. Generally, filtration is more suitable for research scale preparations rather than production scale preparations. Furthermore, a high lipid concentration is necessary for efficient drug encapsulation if liposomes are to be used for drug delivery.

6 Despite substantial research and development in methods for producing liposome (especially MLV) preparations for immunoassay and drug delivery use, there continues to exist a need in the art for new, rapid and simple preparative techniques which are: (1) applicable to liposome formation using widely varying lipid components and concentrations of the same; (2) applicable liposome entrapment of aqueous compositions of widely varying chemical compositions; (3) capable of providing storage stable, substantially  
10 homogeneous populations of small sized liposomes without resort to cumbersome "screening" processes; and (4) susceptible to use in both small and large scale liposome production procedures.

### BRIEF SUMMARY OF THE INVENTION

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The present invention provides novel methods for generating substantially homogeneously-sized populations of aqueous liquid-encapsulating multilamellar lipid vesicles (liposomes) within mean diameter sizes within the range of from 150 to 3,000 nanometers. In brief, one or more lipids, or lipid conjugates, are  
20 suspended in an organic solvent and dried to a thin film on the walls of a vessel. An aqueous liquid to be encapsulated and a collection of spherical contact masses, having a mean diameter of less than 3,000 microns, preferably less than 1,000 microns, and most preferably from 50-100 microns, are then added to the vessel and the vessel is agitated to suspend the lipid film to form a substantially homogeneous population of vesicles having diameters in the range of about 150 to about 3,000 nanometers and preferably  
25 less than 1,000 nanometers.

Presently preferred methods provide for liposomes which may be made from a variety of lipids and lipid conjugates, synthetic surfactants, alone or in combination, with or without membrane stabilizers such as cholesterol, and/or charge modifiers, such as phosphatidic acid, and anti-oxidants such as  $\alpha$ -tocopherol, and the like, over a broad range of lipid concentrations, including the use of small to moderate amounts of  
30 lipid(s) and of marker substances, such as colorimetric or fluorescent dyes, enzymes, or drugs. Both smaller preparations, 1-50 ml, and larger batch preparations, greater than 1 liter, can be prepared by using the appropriate vessel size and agitation apparatus and the process is reproducible from preparation to preparation.

These methods allow for the use of small quantities of marker and lipid, leave no residual solvents, can  
35 involve contact only with glass surfaces, and involve no transfer of liposome preparations from lipid film drying vessels to sizing apparatus. The processes require minimal user attention and the liposomes are not contaminated or damaged during the processes. In addition, the techniques permit control of the liposome size ranges, yield liposomes with efficient encapsulation of marker or drug, and yield liposomes which reproducibly lyse with complement reagent in complement-mediated immunoassays. The liposome size  
40 ranges obtainable are a function of both the agitation time and the size range of beads used. Generally, within the upper limit of use of less than 1,000 micron diameter beads, use of smaller beads for shorter periods of time results in a similarly sized liposome populations as are obtained using larger beads for longer time periods.

In another of its aspects, the invention provides for a dispersion of substantially non-aggregated  
45 multilamellar aqueous liquid-encapsulating lipid vesicles having a mean size distribution ranging from about 150 nanometer to about 3,000 nanometers with presently preferred dispersions of vesicles having a first immunologically active binding pair member (e.g., an antigen) associated with their surfaces and are susceptible to at least 60 percent lysis by excess serum complement in the presence of a stoichiometric amount of a second immunologically active binding pair member (e.g., an antibody).

50 Other aspects and advantages of the present invention will be apparent upon consideration of the following detailed description thereof which includes numerous illustrative examples of practice of the invention.

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### DESCRIPTION OF DRAWING

Figure 1 is a plot of particle mean diameter as a function of time, for liposomes vortexed with different sized glass beads.

## DETAILED DESCRIPTION

The present invention provides methods for the preparation of uniformly sized populations of marker or drug encapsulating liposomes. The following examples illustrate practice of the invention. Example 1 relates to a side-by-side comparison of liposome sizes obtained as a function of the size beads used during preparation and as a function of vortexing time. Example 2 relates to the preparation of vortexed liposomes using a digoxigenin conjugate lipid and entrapping a fluorescent marker. Example 3 relates to the preparation of vortexed liposomes using a digoxigenin conjugate lipid and entrapping an enzyme. Example 4 relates to the preparation of vortexed liposomes entrapping a drug. Example 5 relates to the use of vortexed liposomes in an immunoassay.

The examples which follow are for illustrative purposes and are not intended in any way to limit the scope of the invention.

### EXAMPLE 1

#### Variation in Liposome Size as a Function of Vortexing with Different Sized Glass Beads

A chloroform/methanol (1:1) solution containing egg sphingomyelin (67.5 micromoles), cholesterol (67.5 micromoles) and stearic acid (15 micromoles) was dried to a thin film on the walls of a 50 mL pear-shaped flask by rotary evaporation. Residual solvent was removed under 40 milli-torr vacuum overnight. To the thin film was added 7.5 mL of buffer containing 100 mM NaCl and 50 mM HEPES at pH 7.0. Eighty 3.0 millimeter (3,000 microns) diameter glass beads were added to the flask and the flask was rotated 10 minutes to suspend the lipid film.

Aliquots of these MLVs from the 50 mL flask were transferred to 5.0 mL pear-shaped flasks and vortexed with small glass beads (ranging in diameter from 3,000 to 50 microns in diameter) as follows. To each flask was added 0.5 mL of MLV suspension and 0.87 gms of a pre-selected size range of glass beads, i.e., 3000; 758; 402; 150-210; and 50-100 microns, respectively, in diameter. The flasks were stoppered with glass plugs wrapped with Teflon™ tape and held in place with Parafilm™. Triplicate samples were run for the 3000 microns and 50-100 micron diameter glass beads; for other size ranges single samples were run. A no-beads control was also run. The 5.0 mL pear-shaped flasks were clamped to the platform of a GLASCOL(VB-3) vortexer equipped with a plexiglas adapter designed to hold the flasks firmly by the neck. The samples were vortexed (approximately 3 millimeter excursion) side-by-side at 2000 rpm as measured by a digital tachometer.

Figure 1 shows the changes in the particle mean diameter, as a function of time, for liposomes vortexed with different sized glass beads. Mean diameter was measured by quasi-elastic laser light scattering as calculated by the method of cumulants using a NICOMP Model 200. In the absence of glass beads, vortexing resulted in only a small decrease in mean diameter. The addition of 3000 micron diameter glass beads resulted in smaller diameter liposomes, probably the result of breaking up of the largest particles and aggregates. The use of smaller glass beads, resulted in progressively smaller liposome mean diameters as a function of both vortexing time and glass bead diameter. The triplicate samples prepared using 50-100 micron diameter glass beads showed smaller variance in liposome mean diameter as compared to liposomes prepared using 3000 micron diameter glass beads. The error bars in Figure 1 are plus and minus the standard deviation.

Most of the changes in mean diameter occurred during the first ten minutes of vortexing. When sized populations of large and small liposomes were mixed together in defined proportions, a weighting in favor of the larger particle diameters has been found in analysis by the method of cumulants (Wong, et al., *Biochemistry*, 21:4133-4138 (1982)). The mean diameter in Figure 1 is conservatively interpreted as an upper limit of liposome diameter. The results in Figure 1 are also a function of the size of the vortexing flask, the concentration of lipid, the number of glass beads and the vortexing speed. The selection of the appropriate size of the flask, the concentration of lipid, the number of glass beads, the vortexing speed, and

the vortexing time may vary as a function of the intended use of the liposomes, but will be apparent to a person skilled in the art. The selection of these parameters will be aided by monitoring liposome size distribution, or by monitoring the reproducibility of results obtained in an immunoassay using vortexed liposomes.

## EXAMPLE 2

### Preparation of Vortexed Liposomes Containing a Digoxigenin Conjugate and Entrapping a Fluorescent Marker

Lipid films containing 20, 40, and 100 micromoles of an egg sphingomyelin (47.25%), cholesterol (47.25%), stearic acid (5.0%) and dipalmitoylphosphatidylethanolamine-digoxigenin (0.5%) mixture were prepared on the walls of 10.0 mL pear-shaped flasks, in triplicate, and dried overnight. To each flask was added 2.4 gms of 50-100 micron diameter glass beads and 2.0 mL solution containing 78 mM tris-amide carboxyfluorescein, 50 mM maltose, 0.02% thimerosal and 5mM HEPES, at pH 7.0. The flasks were stoppered, then vortexed side-by-side at 2100 rpm. Samples were removed at 5, 10, and 45 minutes, respectively. The liposomes were aspirated away from the settled glass beads. Buffer was added to wash the beads twice to recover all the liposomes. Any non-encapsulated free fluorophore was removed from the liposome preparation by washing with 9.0 mL of a solution containing 88 mM NaCl, 50 mM maltose, 0.02% thimerosal and 50 mM HEPES at pH 7.0. The liposome preparation was pelleted at 48,000 g for 45 minutes and the supernatant removed. The liposomes were resuspended and the washing procedure repeated a total of 3 times.

Table 1 shows the changes in liposome signal-to-noise, mean diameter, trapped volume, and % capture as a function of lipid concentration and vortexing time.

TABLE 1

CHARACTERIZATION OF VORTEXED LIPOSOMES PREPARED WITH FLUOROPHORE						
	Lipid Concentration (mM)	Vortex Time (Min)	Signal-to-Noise <sup>1</sup>	Mean Diameter (nm)	Trapped Volume (L/Mole)	% Capture
1.	10	5	25	1844	12.5	11.2
2.	10	10	28	1458	13.4	11.1
3.	10	45	35	666	20.9	18.1
4.	20	5	31	2342	10.4	18.1
5.	20	10	29	1648	11.3	22.3
6.	20	45	52	581	18.7	27.9
7.	50	5	39	2513	11.2	53.6
8.	50	10	40	1947	11.2	53.2
9.	50	45	50	822	11.8	47.9

<sup>1</sup>Signal plus noise was measured in the presence of octylglucoside detergent and noise was measured in the absence of octylglucoside. Correction was made for background fluorescence from the detergent and buffer.

As was the case with preparations described in Example 1, mean diameter decreased as a function of vortexing time. Signal-to-noise, trapped volume and % capture increased as a function of vortexing time. The trapped volume decreased and the % capture increased at higher lipid concentrations. The trapped volumes measured for vortexed liposomes prepared with 50-100 micron. glass beads (10.4 - 20.9 liters/mole) were higher than reported by Deamer D. and Oster P (Deamer, et al., "Liposomes" Ch. 1, Liposome Preparation: Methods and Mechanisms; Marc J. Ostro (Ed.) M. Dekker, Inc., pg. 2751 New York, New York (1983)) for multilamellar liposomes (2-7 liters/mole). The high % capture at higher lipid concentrations, together with the decreasing mean diameter at longer vortexing times, is an advantage in potential drug delivery applications.

EXAMPLE 35 Entrapping an Enzyme in Liposomes Vortexed with 50-100 micron Diameter Glass Beads

A ten micromole lipid film (4.725 micromoles egg sphingomyelin, 4.725 micromoles cholesterol, 0.5 micromole stearic acid, 0.05 micromoles dipalmitoylphosphatidylethanolamine-digoxigenin) was dried on the walls of a 5.0 mL pear-shaped flask.

10 To the flask were added 0.5 mL of a 20 mg/mL solution of alkaline phosphatase (33,400 Units) in buffer containing 100 mM NaCl, 50 mM trehalose, 1 mM MgCl<sub>2</sub>, 0.1 mM ZnCl<sub>2</sub>, 50 mM HEPES at pH 8.0 and 0.6 gms of 50-100 micron diameter glass beads. The flask was vortexed at 2000 rpm for 50 minutes. Unencapsulated free alkaline phosphatase was removed by centrifuging the liposomes at 45,000 g for 30 minutes minutes and removing the supernatant. The liposomes were resuspended in a buffer containing  
15 100 mM NaCl, 50 mM trehalose, 6 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 50 mM HEPES at pH 8.0. The wash step was repeated a total of 4 times.

The ratio of enzyme activity encapsulated versus free enzyme, i.e., unencapsulated, was determined by lysing an aliquot of liposomes with octylglucoside detergent. Reaction rates were measured at 37°C in the presence of 8 mM p-nitrophenylphosphate substrate. The signal-to-noise was 40.8 and the percent of  
20 enzyme encapsulated (measured as enzyme activity) in the liposomes was 98%.

EXAMPLE 4

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Entrapping a Drug in Liposomes Vortexed With 50-100 Micron Diameter Beads

A 120 micromole lipid film (54 micromoles egg sphingomyelin, 54 micromoles cholesterol, 12 micromoles stearic acid) was dried on the walls of a 10 mL pear-shaped flask. To the flask was added 6.0 mL of the drug urokinase dissolved in 0.9% saline (120,000 IU/mL) and 3 gms of 50-100 micron diameter glass beads. The flask was stoppered and vortexed for 60 minutes at 2000 rpm. The mean diameter of the liposome suspension was 1509 nanometers. External urokinase was removed by pelleting the liposomes by centrifugation and resuspending the liposomes in buffer.

35

EXAMPLE 5

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Use of Vortexed Liposomes In an Immunoassay

Digoxigenin-liposomes for use in an immunoassay to detect digoxin were prepared by vortexing a 10 micromole lipid film, having the same composition and fluorophore as described in Example 2, with 50-100 micron diameter glass beads for 45 minutes. The digoxigenin conjugated liposomes comprise the first immunological binding pair member and the digoxin to be assayed comprises the second immunological binding pair member. Rabbit anti-digoxin anti-sera was diluted in the buffer of Example 2 plus 288 mM MgCl<sub>2</sub> so as to give 35% of the maximum liposome lysis obtained with 10% octyl glucoside with the 0.0 ng/mL standard. The complement source was seventy percent horse plasma in buffer and the standards  
45 included DIGOXIN II FPIA calibrators 9611-01 lot #06998 A2, Abbott Labs, North Chicago.

The assay protocol consisted of the following steps, at the indicated time intervals, at 37°C:

- i) add 25 microliters sample to 45 microliters 0.1 N HCl and incubate for 30 seconds; then add buffer to give a final sample dilution of 1:88;
- ii) add 45 microliters antisera to 365 microliters sample, diluted 1:88, and incubate for 5 minutes;
- 55 iii) add 500 microliters buffer containing 625 picomoles liposomes and incubate for 10 minutes;
- iv) add 60 microliters complement to 60 microliters of the incubate of step iii) and incubate for 10 minutes; dilute this mixture 1:13 with buffer; and

v) read the fluorescence intensity at excitation: 490 nm and emission: 520 nm.

Table 2 shows a digoxin standard curve generated using vortexed liposomes, on an automated instrument programmed to perform the reagent additions and to read fluorescence. These results indicate an inverse relationship of the fluorescence intensity and the amount of digoxin in the sample.

TABLE 2

DIGOXIN STANDARD CURVE			
Calibrator Standard (ng/mL)	Fluorescence Intensity	Mean	CV (S.D/Mean) (%)
0.0	4838 4963 4882 4819	4876	1.14
0.5	4490 4256 4235 4278	4315	2.38
1.0	3898 4025 3983	3969	1.34
2.0	3288 3301 3259	3283	0.54
3.0	2744 2677 2858	2759	2.68
5.0	1845 1616 1675	1645	1.47

The foregoing illustrative examples relate to the preparation of sized populations of liposomes. While the present invention has been described in terms of specific methods and compositions, it is understood that variations and modifications will occur to those skilled in the art upon consideration of the present invention.

For example, it is envisioned that mixtures of large and small diameter vortexing spheres, such as greater than and less than 1,000 microns in diameter, may be equally effective. Also, while the use of glass beads has been described, it will appear that other non-glass vortexing spheres, such as metal or ceramic or the like, may be employed in the practice of the invention. Moreover, while specific vortexing times and speeds have been described, the use of other vortexing times and speeds may readily be used according to the invention. The use of various other glass and non-glass vortexing vessels of other shapes and volumes, other than those flasks specifically described, may be equally effective.

Similarly, while lipid films have been described comprised of sphingomyelin, cholesterol, stearic acid, and dipalmitoylphosphatidylethanolamine-digoxigenin, other lipids and lipid conjugates such as phosphatidylcholine, phosphatidylethanolamine, various glycolipids, single-chain lipids, fatty acids, dialkyl-type synthetic surfactants, and the like may be used alone or in combination. These components may contain membrane stabilizers in addition to cholesterol, such as cholestanol and the like and/or charge modifiers, such as phosphatidic acid, dicetylphosphate, stearylamine, and anti-oxidants such as  $\alpha$ -tocopherol, over a broad range of concentrations.

In addition, other enzymes can be encapsulated other than urokinase, such as alkaline phosphatase,  $\beta$ -galactosidase, and  $\beta$ -lactamase. Other fluorophores, such as calcein, carboxyfluorescein, n-methyl glucamide carboxyfluorescein, and chromophores such as sulforhodamine B may also readily be used.

Similarly chemiluminescent markers, radioisotopes, contrast agents, paramagnetic or NMR detectable agents can be incorporated.

Numerous modifications and variations in the invention as described in the above illustrative examples are expected to occur to those skilled in the art and consequently only such limitations as appear in the appended claims should be placed thereon. Accordingly, it is intended in the appended claims to cover all such equivalent variations which come within the scope of the invention as claimed.

## Claims

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1. A method for the preparation of a substantially homogeneous population of aqueous liquid encapsulating multilamellar lipid vesicles having mean diameters within the range of from about 150 to about 3,000 nanometers, said method comprising the steps of:  
 adding to a vessel one or more lipids in an organic solvent;  
 15 drying and lipids to a thin film onto the walls of said vessel;  
 adding to said vessel said aqueous liquid and a collection of contact masses having mean diameters within the range of from 50 to 3,000 microns; and  
 agitating said vessel to form said multilamellar vesicles.

2. The method according to claim 1, wherein said collection of contact masses are spherical glass beads and wherein the mean diameter of said glass beads is selected from the group consisting of 3,000 micron, 758 micron, 402 micron, 150-210 micron, and 50-100 micron diameters.

3. The method according to claim 1, wherein agitation of said vessel comprises (1) vortexing at greater than 100 rpm for greater than 1 minute or (2) vortexing at 2,000 rpm for about 45 minutes.

4. The method according to claim 1, wherein said lipid is a member selected from the group consisting of phospholipids, sphingolipids, glycolipids, fatty acids, sterols, spingomyelin, cholesterol, stearic acid and dipalmitoyl phosphatidylethanolamine-digoxigenin.

5. The method according to claim 1, wherein the ratio of said lipid, in moles, to the volume of said vessel, in milliliters, to the mass, in grams, of said collection of contact masses is 20:10:24.

6. The method according to claim 1, wherein said aqueous liquid to be encapsulated comprises a member selected from the group consisting of buffer, fluorophores, enzymes, and drugs.

7. The method according to claim 6, wherein said aqueous liquid is selected from the group consisting of tris-amide carboxyfluorescein and urokinase.

8. A dispersion of substantially homogeneously sized lipid vesicles having mean vesicle diameters within the range of from about 150 to about 3,000 nanometers for use in a diagnostic immunoassay or in drug delivery.

9. The dispersion of lipid vesicles according to claim 8, wherein said vesicles have a first immunological binding pair member associated with their surfaces and are susceptible to at least 60% lysis by excess serum complement in the presence a stoichiometric amount of a second immunological binding pair member.

10. The dispersion of lipid vesicles according to claim 9, wherein said first immunological binding pair member is phosphatidylethanolamine-digoxigenin.

11. A method for the preparation of a substantially homogeneous population of aqueous liquid encapsulating multilamellar lipid vesicles having mean diameters within the range of from about 150 to about 3,000 nanometers, said method comprising the steps of:

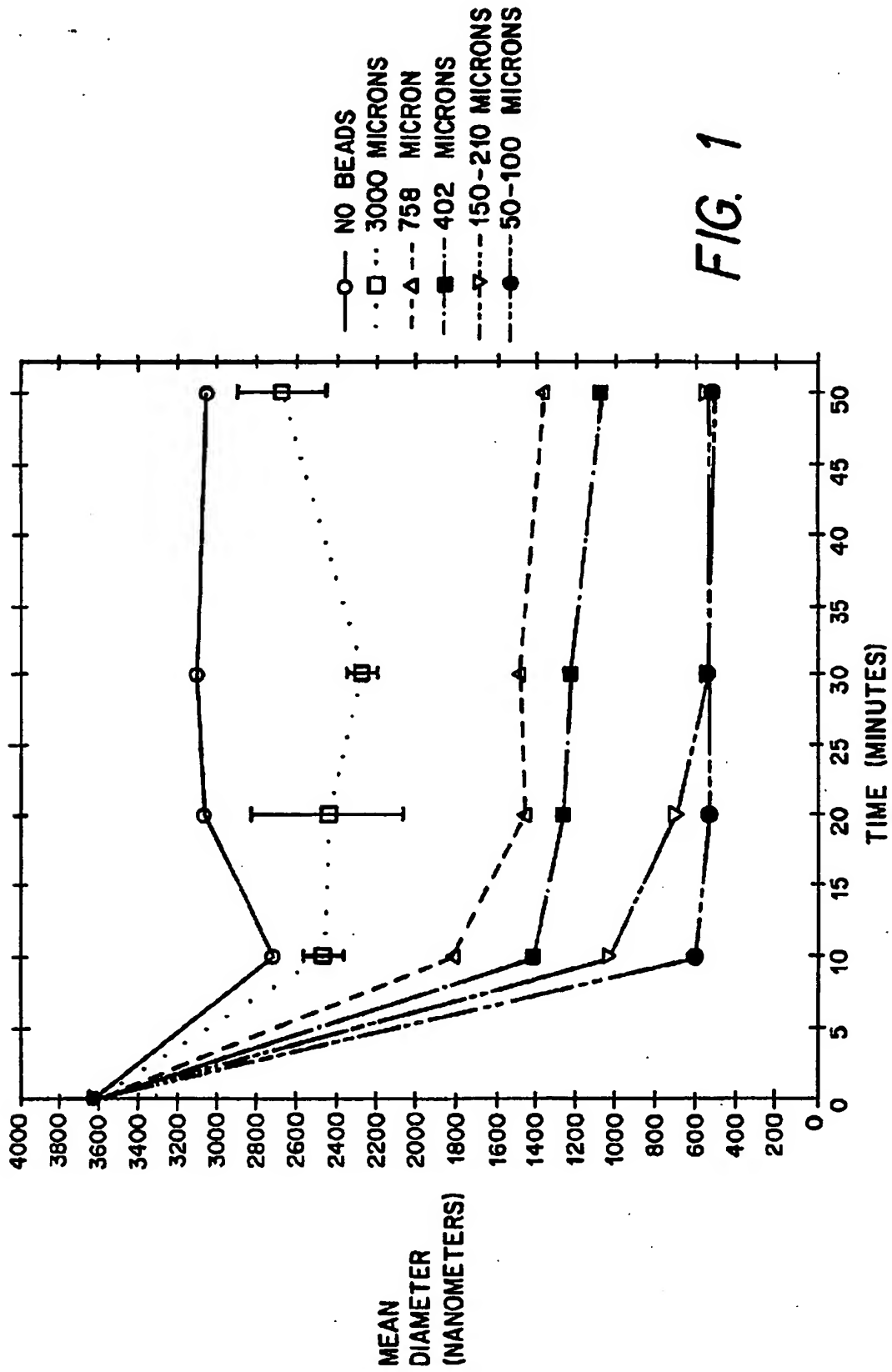
45 adding to a first vessel one or more lipids in an organic solvent;  
 adding to said first vessel said aqueous liquid and a first collection of contact masses;  
 agitating said first vessel to form a first collection of multilamellar vesicles; and  
 agitating said first collection of multilamellar vesicles in the presence of a second collection of contact masses to form a second collection of multilamellar vesicles of the desired size.

50 12. The method according to claim 11, wherein said first collection of contact masses are spherical glass beads of about 3,000 microns in diameter.

13. The method according to claim 11, wherein said second collection of masses are spherical glass beads and wherein the mean diameter of said glass beads is selected from the group consisting of 3,000 microns, 758 micron, 402 micron, 150-210 micron, and 50-100 micron diameters.

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European Patent  
Office

# EUROPEAN SEARCH REPORT

Application Number

EP 88 11 8298

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
A	EP-A-0 119 020 (E.R. SQUIBB & SONS) * Page 4, line 1 - page 7; pages 11-13; examples 1-4; claims 1-6 * ---	1-13	A 61 K 9/50 G 01 N 33/543
A	EP-A-0 186 352 (TECHNICON INSTRUMENTS CORP.) * Page 6, line 7 - page 13; pages 17-19; example 1; claims * ---	1-13	
A	DE-A-3 335 701 (LIPODERM PHARMACEUTICALS LTD) * & US-A-4 485 054 (CAT.D) * -----		
			TECHNICAL FIELDS SEARCHED (Int. Cl.4)
			A 61 K
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 03-03-1989	Examiner BENZ K.F.
CATEGORY OF CITED DOCUMENTS			
X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document		T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons ----- & : member of the same patent family, corresponding document	